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13. ABSTRACT (Maximum 200 Words)

This report describes the research accomplishments/findings covering the above indicated period and outlined in the approved statement of work. In short, we have locally manufactured, tested and successfully used the spotted cDNA microarray chips to analyze gene expression profiles of E1A induced primary mouse fibroblasts either wild-type or deficient for the ARF and/or p53 genes. We found that the E1A oncoprotein regulates the expression of a myriad of targets involved in a diversity of functions such as apoptosis, cell cycle progression, checkpoint control, DNA replication, angiogenesis, biosynthesis, as well as structural and cytoskeletal elements. Our results revealed the pleiotropic role of E1A and produced a databank of potential apoptotic targets that can be studied and characterized. Following up on some targets, we demonstrated that E1A can coordinately up-regulate caspase expression through E2F via a direct transcriptional mechanism, providing therefore an example of how apoptosis can be 'hardwired' to cell-cycle progression. Importantly, we also showed that, p53-generated signals resulting in caspase activation (for example, cytochrome *c* release from mitochondria) cooperate with p53-independent increases in caspase expression to induce apoptosis, implying that oncogene-induced apoptosis proceeds through a highly coordinated series of events.

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Introduction

Among the most significant advances of cancer research during the last decade is the realization that apoptosis has a profound effect on the malignant phenotype. In our proposal, the overall goal is to identify and study new targets involved in oncogene-induced apoptosis. Specifically, we sought to (i) identify the common and separate ARF and p53 targets involved in the oncogenic stress pathway (ii) characterize the genes identified in (i) and (iii) investigate the *in vivo* validity and physiological implications of the ARF/p53 targets. We combine two powerful technologies in our analysis. The first involves a highly comparable populations of primary cells developed in our lab. These cells only differ in their expression of specific genes (ARF and p53). The second technology is a gene expression profile analysis using microarrays. By comparing gene expression profiles we expect to determine in an unbiased approach the behavior of apoptotic/oncogenic targets and determine whether ARF acts solely through p53 or engages other pathways. Our analysis will survey thousands of genes and hope to provide new insight into the oncogene-induced apoptotic network. Such insights will help understand the molecular details of breast cancer and assist in developing therapeutic strategies against the disease.

Body

In the approved statement of work, we stated that our objectives for the first tier of the award period is to perform gene expression profile analyses on E1A infected primary fibroblasts and identify targets involved in the oncogenic stress pathway. We proposed achieving this goal by:

i) manufacturing spotted cDNA chips on-site and performing the quality controls necessary (Task 1, part A & B)
ii) generating Mouse Embryo Fibroblasts (MEFs) with different genetic backgrounds (Task 1, part C)
iii) introducing the E1A oncogene using retroviral-mediated gene transfer techniques into MEFs and extracting and labeling RNA for microarray analysis (Task 1, part D & E)
iv) performing the hybridization, extracting the intensities, and statistically curating and analyzing the expression data (Task 1, part F)

Here, we summarize the research accomplishments associated with each task. A full description of the research work emanating from the microarrays is presented in the appended publication (Appendix A). Also, experimental design pertinent to the manufacturing of the microarray chips is covered in the appended presentation (Appendix B).

- *Establishing the microarray technology and the tools for data analysis (Task 1, part A & B)*:

To characterize genome-wide changes in transcription that accompany E1A infection in MEFs, we used DNA microarrays (manufacturing steps are described in appendix B) containing around 15,000 cDNAs and ESTs obtained from the National Institute of Aging collection. Crude estimations (considering redundancy in the collection) suggest that they represent 60 % of all the mouse transcription units (excluding spliced forms). We have optimized all steps in the array process including sample preparation, DNA labeling, probe hybridization as well as intensity, sensitivity, specificity, and background reduction. The current procedure uses fluorescing cyanine dyes (Cy3 and Cy5, Amersham) to label reactive aminoallyl groups incorporated on single stranded DNA. Labeled molecules are subsequently hybridized on the glass chips at optimal conditions. Fluorescent intensities are retrieved by a laser-excitation-based epifluorescence scanning system (GerePix 4000, Axon Instruments, Inc.). Then, data are extracted by superimposing a grid over each array using GenePix3.0 software (Axon Instruments, Union City CA). Spots of poor quality, determined by visual inspection, are removed from further analysis. Data collected for each array are analyzed (described below) and stored in our databases.

Fluorescently labeled cDNAs from E1A infected MEFs are hybridized against labeled cDNAs from MEFs infected with a vector control to spotted cDNA chips. Four replicate hybridizations (including dye-swaps or color reversal) are performed for each perturbation (e.g. Figure 1-d). To allow for direct comparison of the behavior of all genes in response to all the perturbations, we performed the experiments under well-defined condition of temperature, growth media, and cell density. All duplicate experiments are simultaneously hybridized washed, scanned using

identical conditions and reagents. We also perform "self-to self" hybridization, where the same sample are labeled separately with Cy5 and Cy3 and hybridized (Figure 1-c). Ideally, the ratio of Cy5 to Cy3 for all the probes would be 1. Consistantly, in our self to self-experiments, we observe scatter about the ratio of 1, with very few ratios (<0.02%) observed to be >2.

We import raw data files generated by the scanner software (Axon's GenePix3 in our case) to our databases for further analysis. For normalization, features for which R^2 values are above 0.4, and 50% of the feature pixels are 1 standard deviation (SD) above background pixels in both (635nm and 532nm) channels are considered. All these values have been empirically defined and adjusted. Data sets qualifying our parameters for at least 80% of the samples in a given time course are used for further analysis. Data points that do not meet these criteria are left blank in the primary data tables. Cy5/Cy3 are computed for each data point, where (Cy5/Cy3) is the median pixel intensity normalized ratio of the background-corrected intensities.

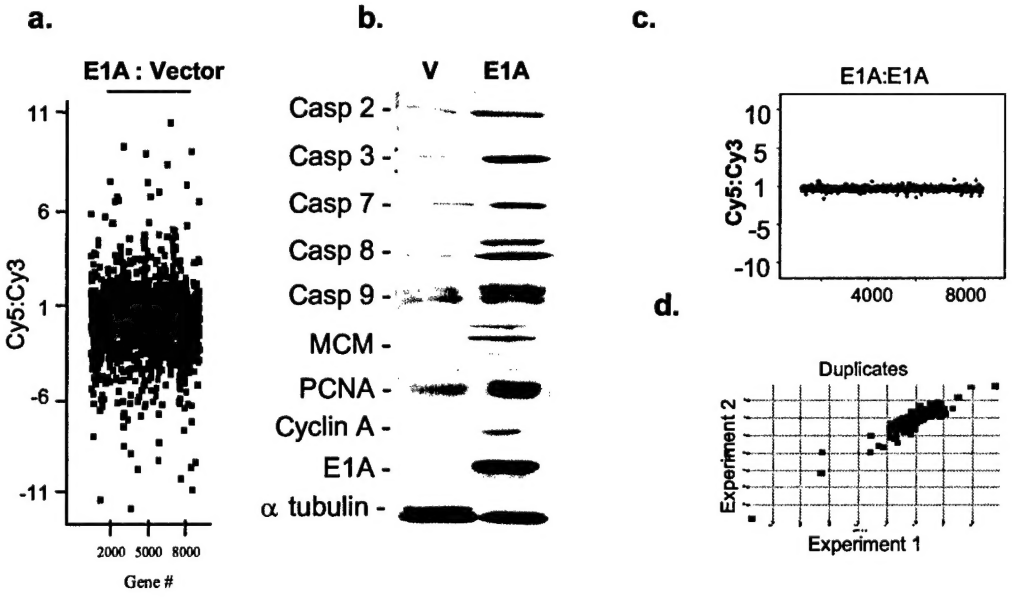
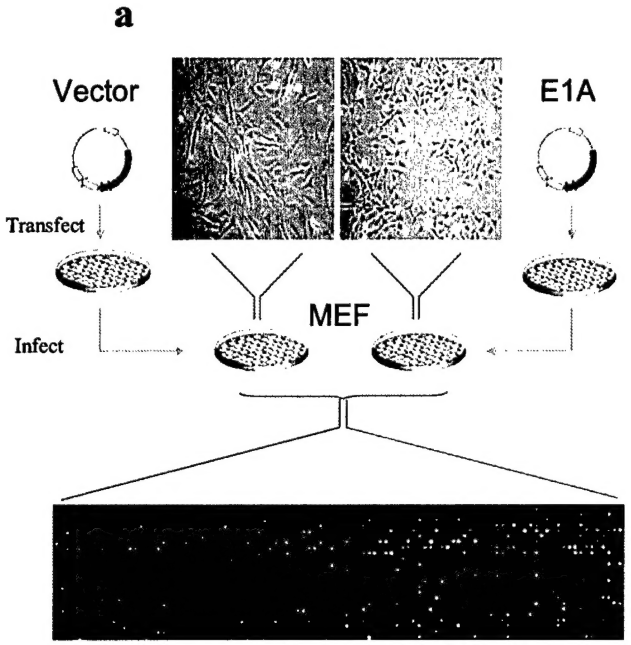
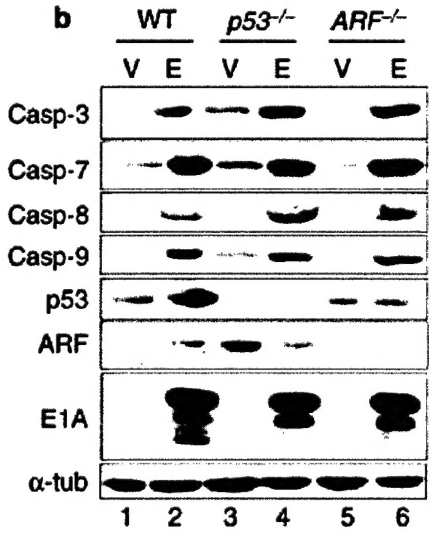


Figure 1 a. Scatter plot depicting the gene expression profile of 15,000 genes and ESTs in wild type MEFs transduced with E1A. E1A samples were labeled with the red Cy5 dye and Vector controls labeled with the green Cy3 dye. The y-axis represents the Cy5:Cy3 ratio (only ratios between 12 and -12 are shown for simplicity). The x-axis represent the gene index. Data in scatter plots represents analysis after GenePix, S-plus and S potfire treatment. b. Validation of a selection of E1A targets (Caspases, MCM, PCNA, Cyclin A) by western blot analysis. c. Self to self controls are performed to verify equal dyes incorporation as well as to detect the inherent level of noise in the application. The same amount of cDNA is labeled with Cy5 and Cy3 respectively and samples combined and let hybridized at optimal conditions. d. Ratios from duplicated experiments are plotted on the x- and the y-axis respectively. Data points align along a slope of 1, representing high degree of reproducibility among chips.

- generation of MEFs in different genetic backgrounds and retroviral infection (Task 1, c and d):

Primary Mouse Embryonic Fibroblasts (MEFs) from wild- type, p53-/-, ARF-/-, Rb-/-, p53-/, and p53-/- ARF-/- mice were isolated and cultured as described in reference 1 and figure 2.





PS: Full description of the reseach work results pertinent to the model in part c is presented in the form of primary publication in appendix A (Nahle *et al. Nature cell biology 2002)*

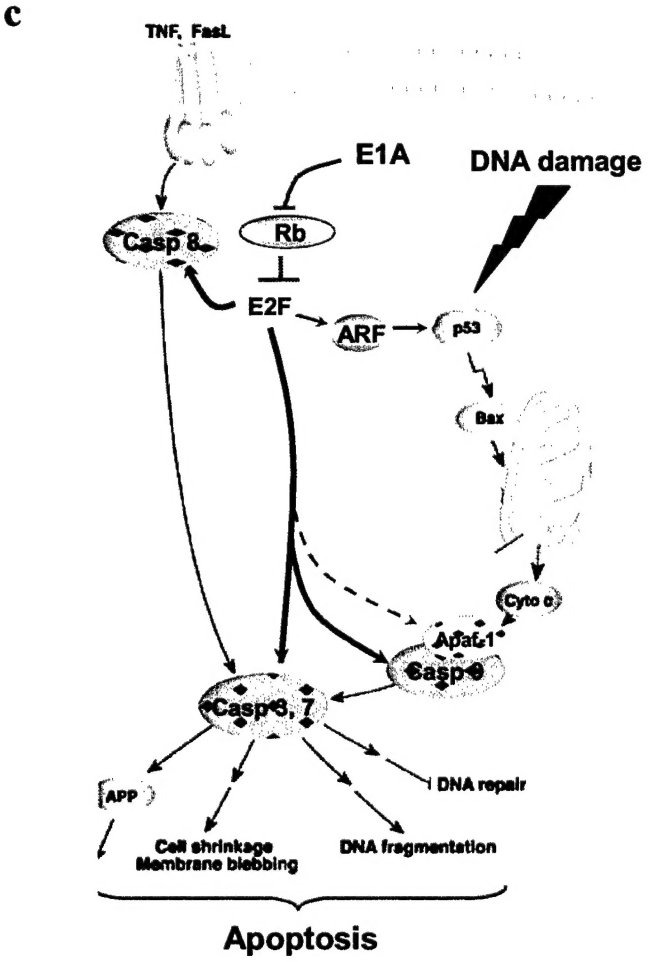


Figure 2 a. Schematic representation of the retoviral mediated transfer technique proposed in the statement of work depicting photomicrographs of E1A and vector infected MEFs. b. The levels of caspase proenzymes as well as ARF and p53 were assessed in MEFs (wild-type, *p53*$^{-/-}$ and *ARF*$^{-/-}$). Cells transduced with a control retrovirus (V) or a retrovirus expressing *E1A* (E) by immunoblotting using antibodies directed against the indicated proteins. Tubulin was used as a loading control. MEFs generated locally were used c. A model for oncogene-induced apoptosis involving both p53-dependent and p53-independent pathways. E2F signaling indirectly to p53 facilitates caspase activation and simultaneously produces increases in caspase expression. These two pathways function in concert to ensure efficient cell death.

- *Computational softwares and Microarray Results (Task 1, f)* :

We use several specialized softwares in the analysis. Data acquisition and initial quality control operations are performed using GenePix™ (Axon Instruments, Inc.). GenePix is a fully featured array acquisition and first-pass analysis software package. It contains advanced imaging and data evaluation capabilities. Using GenePix, intensities are converted into numerical quantification and local background effects are automatically calculated and subtracted. Through GenePix we also export intensities, ratios and quality measure data for analysis with advanced information systems. S-plus (Insightful Corporation, CA) is a mathematical and statistical software package and one of those systems. Scripts and algorithms have been developed by Drs. M. Wigler and V. Mittal and incorporated in S-plus. These scripts perform effective operations on data normalization and filtering of data points below threshold intensities. Importantly, spatial biases on individual chips can also be detected using the S-plus tools. Spatial biases often distort microarray data and are often generated from differential stability of fluorescent dyes, generation of local thermal gradients during micro-hybridization under a glass coverslip or robotic printing of arrays. An additional step in our data analysis is the use of Spotfire DecisionSite™ 7.0 (Spotfire, MA). Here, data from several experiments already analyzed using S-plus are merged and visualized using this graphic interface program. This program enables us also to interface with web-based options for pathway interrogation, gene functional assignment, and compound analysis. In short, the data analysis is rigorous and designed to extract the outmost from the array results with accuarcy and confidence.

Using the procedure described above, we have conducted a large number of control experiments to determine whether we can obtain meaningful data. A standard control procedure is to swap the labeling dyes Cy3 and Cy5 among experimental samples and hybridize under the same conditions. As expected, a reversed Cy5 to Cy3 ratio has been consistently observed in such color reversal controls (appendix B). We also label each cDNA preparation with both Cy5 and Cy3 dyes and hybridize in a self to self procedure (Figure 1-c and appendix B). In such experiments the Cy5:Cy3 ratios of all genes are closely tight around a ratio of 1 (Figure 1-c), providing confidence with the labeling procedures and the probe preparation. Also, a broad dynamic range of fluorescence sensitivity can be detected on the chips as well as a high level of inter-chip reproducibility in duplicate experiments (Figure 1-d). Also, as internal controls, numerous housekeeping genes and replicated clones are randomly dispersed on the chip (data not shown). And, results from replicated genes indicate, as expected, nearly identical values suggesting an intra-chip reproducibility independent of spots geometric location. The gene expression analysis we performed on E1A expressing cells in wt, p53-/-, ARF-/- and ARF-/-p53-/- backgrounds (unpublished, only wild-type MEFs are shown in Figure 1) provides clear evidence of the pleiotropic role played by the E1A oncogene. We found that the E1A oncogene regulates the expression of a myriad of targets involved in a diversity of functions such as apoptosis, cell cycle progression, checkpoint control, DNA replication, angiogenesis, biosynthesis, as well as structural and cytoskeletal elements. Standing alone, this study represents the first report that depicts the effect of E1A on mammalian cells gene expression as a simultaneous and global readout. Hence, revealing the pleiotropic role of E1A and producing a databank of potential apoptotic targets that can be studied and characterized.

The Follow us study on the regulation of caspases by E1A and E2F is presented (as a publication) in details in Appendix A.

Key Research Accomplishments

- Accomplishments related to establishing the microarray and cell system technologies:

  - Manufacturing of the 15,000 gene chip (gene library source: National Institute of Aging)
    1. Replication of genes, PCR amplification, and purification of PCR products
    2. Spotting of clones on polylysine coated glass slides using CSHL arrayer
    3. Testing the quality of spotting and hybridization
  - Generation of mice with different genotypes to obtain p53-/-, ARF-/-, p53-/- ARF-/-, and Rb-/- embryos ; confirmation of genotypes, and isolation of Mouse embryo Fibroblasts (MEFs) from desired genetic backgrounds.
  - Introduction of the E1A oncogene using retroviral-mediated gene transfer system into isolated MEFs.
  - Extracting mRNA from exponentially growing MEFs, labeling with cyanine dyes (cy3 and cy5), and hybridization on cDNA chips.
  - Extraction of fluorescent intensities, normalization, threshing and statistical analysis of data.

- Accomplishments using the technology:

  we discovered that:

  - The adenovirus E1A induces caspases protein and transcript levels
  - Caspase induction is independent of ARF and/or p53
  - Caspase regulation is controlled by the retinoblastoma gene product Rb
  - Caspase promoters contain predicted E2F binding sites
  - Caspase 7 is a direct E2F target
  - The Rb/E2F and the ARF/p53 pathways cooperate to induce cell death by inducing complementary apoptotic triggers

Reportable Outcomes

- *Degrees:*

  Ph.D. in Physiology and Biophysics, SUNY at Stony Brook, May 2003

- *Primary papers:*

  Z. Nahle, J. Polyakoff, R V. Davuluri, M E. McCurrach, M D. Jacobson, M. Narita, M Q. Zhang, Y. Lazebnik, D. Bar-Sagi, and S W. Lowe. *Direct coupling of the cell cycle and cell death machinery by E2F.* ***Nature Cell Biology,*** Vol 4: 28-33, Nov. ***2002.***

- *Abstracts and poster presentations (international meetings only):*

  Z. Nahle, M. McCurrach, J. Polyakoff, R .Davuluri, M. Zhang, J. Pelletier, Y. Lazebnik, and S. Lowe. *Oncogenic Induction of Caspases via an ARF-p53 Independent Pathway*. **Programmed Cell Death** (November 9-11, **2001**) P.186, Cold Spring Harbor Laboratory, New York.

  Z. Nahle. *Cell Cycle and Cell Death*. **The Cell Cycle as a Target in Chemoprevention and Cancer Therapy** (October 3-4, **2002**, pp.32) Holiday Inn - Town Lake Austin, Texas.

  Z. Nahle, M. McCurrach, R. Davuluri, M. Jacobson, J. Polyakoff, M. Zhang, Y. Lazebnik, D. Bar-Sagi and S. Lowe. E2F-1 Directly Regulates Caspases Coupling Cell Cycle to Cell Death. **Cancer Genetics & Tumor Suppressor Genes** (August 14-18, **2002**, pp.293) Cold spring Harbor Lab, NY.

  Z. Nahle , R. Davuluri , M. McCurrach , J. Polyakoff , M. Jacobson ,M. Zhang , Y. Lazebnik , D. Bar-Sagi and S. Lowe. *Direct Coupling of Cell cycle Progression and Apoptosis by E2F-1*. **Apoptosis and Cancer: Basic Mechanisms and Therapeutic Opportunities in the Post-Genomic Era** (February 13-17, **2002**, B.17), Hilton, Hawaii.

- *Oral presentations (international meetings only):*

  Cancer Genetics & Tumor Suppressor Genes, Grace Auditorium, Cold spring Harbor Lab, New York, August 2002.

  The Cell Cycle as a Target in Chemoprevention and Cancer Therapy, Holiday Inn, Texas, October **2002**.

- *News & Media reports:*

  Featured in HIGHLIGHTS *Nature Reviews Cancer*. 2: 892, Dec 2002 (Appendix C).
  Featured in HIGHLIGHTS *Nature Reviews Mol. Cell. Biol.* 3: 889, Dec, 2002 (Appendix C).
  Featured in Biology Reports Ltd Faculty *of* 1000, 2002 .
  Featured in "*the longislander*" newspaper, No.7, pp 18, Oct. 10 2002 (Appendix C).

Conclusions

In conclusion, the generous support provided by the BCRP fellowship has enabled us to execute, on schedule, the research plans outlined in the approved statement of work. We have manufactured, tested and successfully used the spotted cDNA microarray chips as originally intended. Thus far, our findings have lead us to propose a plausible mechanism explaining how oncogene-induced apoptosis limits the transforming potential of excessive mitogenic signaling. The model has been published in the prestigious Journal Nature *cell biology* underscoring the novelty and impact of the work. In addition, our research work has been highly cited and selected for the 'Highlights' section of Nature Reviews and was reported as 'must read' publication in the Faculty *of* 1000.

- So what ! (evaluating the knowledge as a scientific and medical product)

Oncogene-induced apoptosis limits the transforming potential of excessive mitogenic signaling, but precisely how cells 'sense' aberrant proliferation has been unclear. In our DOD-BCRP supported studies, we have shown that this can be achieved, in part, by coupling cell-cycle progression to caspase expression through E2F. Hence, E2F transmits mitogenic signals to the cell-cycle machinery by directly regulating the transcription of genes required for S phase entry and simultaneously upregulates the cell death machinery through a similar mechanism. As a consequence, cycling cells are primed for apoptosis should proliferation be perceived as aberrant. Together, our results demonstrate that oncogenes can coordinately upregulate caspase expression through a direct mechanism, providing an example of how apoptosis can be 'hardwired' to cell-cycle progression.

We showed that deregulation of the Rb–E2F pathway by E1A, loss of Rb or enforced E2F expression coordinately increases caspase expression, at least in part, through a direct transcriptional mechanism. Although not sufficient for apoptosis, high levels of signalling caspases may increase the probability that a death-inducing signal overcomes endogenous caspase inhibitors (for example, inhibitor of apoptosis (IAP) proteins) to trigger a caspase cascade, whereas high levels of effector caspases may ensure that apoptosis proceeds efficiently once initiated. Hence, p53-generated signals resulting in caspase activation (for example, cytochrome *c* release from mitochondria) cooperate with p53-independent increases in caspase expression to induce apoptosis. Alternatively, increases in caspase expression — particularly Casp-8 — may sensitize cells to death-inducing ligands. Together, our results explain the ability of E2F-1 to cooperate with p53 in apoptosis and the increased sensitivity of oncogene-expressing cells to pro-apoptotic cytokines, such as TNF-alpha. More importantly, they imply that oncogene-induced apoptosis proceeds through a highly coordinated series of events, ensuring that cell death is efficiently executed.

References

1- Serrano, M., Lin, A.W., McCurrach, M.E., Beach, D. & Lowe, S.W. Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell* 88, 593-602 (1997).

2- Nahle, Z., *et al.*, Direct coupling of the cell cycle and cell death machinery by E2F. *Nature cell biology*, 4(11), 59-64 (2002).

Direct coupling of the cell cycle and cell death machinery by E2F

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Unrestrained E2F activity forces S phase entry and promotes apoptosis through p53-dependent and -independent mechanisms. Here, we show that deregulation of E2F by adenovirus E1A, loss of Rb or enforced E2F-1 expression results in the accumulation of caspase proenzymes through a direct transcriptional mechanism. Increased caspase levels seem to potentiate cell death in the presence of p53-generated signals that trigger caspase activation. Our results demonstrate that mitogenic oncogenes engage a tumour suppressor network that functions at multiple levels to efficiently induce cell death. The data also underscore how cell cycle progression can be coupled to the apoptotic machinery.

Excessive mitogenic signalling is coupled to apoptosis as part of a fail-safe mechanism that suppresses oncogenic transformation[1]. For example, both the E1A and Myc oncoproteins induce ARF (the alternative reading frame product of the *INK4a/ARF* tumour suppressor locus), which in turn activates p53 to trigger cell death[2]. As a consequence, disruption of the ARF–p53 pathway allows inappropriate proliferation and survival, thereby stimulating tumorigenesis[2,3]. However, oncogenes can also signal to p53 through ARF-independent mechanisms[4–6] and promote apoptosis in ways that are entirely independent of p53 (ref. 1).

The Rb tumour suppressor is another factor that regulates proliferation and survival[3,7]. Rb restrains proliferation, in part, by modulating the activity of E2F transcription factors. In quiescent cells, Rb associates with several E2Fs, resulting in the repression of proliferation-associated genes. As cells progress into the cell cycle, cyclin-dependent kinases phosphorylate Rb, freeing E2F and allowing it to directly transactivate genes required for S phase entry. As a consequence, loss of Rb promotes aberrant proliferation. Disruption of Rb also promotes apoptosis and enforced expression of E2F can induce apoptosis through both p53-dependent and -independent mechanisms[1,3]. In contrast to how E2F promotes S phase entry, however, most studies suggest that E2F controls apoptosis though an indirect mechanism. For example, E2F can transcriptionally activate ARF or p73 (a member of the p53 family), which in turn produces secondary changes in gene expression that eventually trigger apoptosis[8–11].

Caspases are essential components of the apoptotic machinery[12]. These proteases are synthesized as inactive proenzymes and processed to an active state during apoptotic cell death. Initiator caspases (for example, Casp-2, -8 and -9) trigger a cascade that results in activation of the effector caspases (for example, Casp-3 and -7), which in turn produce the characteristic morphological changes associated with apoptosis[12]. The 'extrinsic' apoptotic pathway is triggered by interactions between cytokines and death receptors, resulting in activation of Casp-8. The 'intrinsic' pathway can be initiated by changes to the mitochondria that facilitate the release of cytochrome *c*, which associates with Apaf-1 and Casp-9 to promote caspase activation. How oncogenic signals interact with the apoptotic machinery is poorly understood, but it may involve changes to both pathways[1,13,14].

Results

E1A induces caspases in an ARF- and p53-independent manner. To determine whether oncogenes could influence caspase expression, we examined the effect of the *E1A* oncogene on caspase expression. E1A was introduced into early passage mouse embryo fibroblasts (MEFs) and normal diploid human fibroblasts (IMR90) by retroviral-mediated gene transfer. Infected cells were harvested for immunoblotting with antibodies specific for initiator (Casp-8 and Casp-9) or effector (Casp-3 and Casp-7) caspases. In both human and murine cells, E1A expression induced a 5–15-fold increase in the expression of all caspases examined (Fig. 1a, compare lanes 1 and 2 with lane 3 and 4). Similar results were also observed for Casp-2 (data not shown). Therefore, E1A coordinates the upregulation of multiple caspases, perhaps through a common mechanism.

E1A can promote apoptosis through the ARF–p53 tumour suppressor pathway, and p53 directly regulates expression of Apaf-1 (refs 15–17), a cofactor of Casp-9. To determine whether ARF and/or p53 also regulate caspase expression in response to oncogenes, we investigated whether these proteins are required for E1A-induced caspase expression. E1A induced expression of all four caspases in cells deficient in either p53 or ARF (Fig. 1b, compare lane 2 with lanes 4 and 6). Therefore, E1A regulates caspase expression through a p53-independent mechanism.

E1A induces caspases by inactivating Rb and deregulating E2Fs. Rb is an essential target of E1A in apoptosis; furthermore, E1A mutants that are unable to inactivate Rb are defective at inducing ARF and p53, and promoting cell death[15,18]. These E1A mutants also failed to increase caspase levels (data not shown). To determine whether inactivation of Rb is sufficient to increase caspase expression, we examined caspase levels in MEFs derived from *Rb*-deficient mice ($Rb^{-/-}$). Indeed, $Rb^{-/-}$ MEFs expressed much higher levels of Casp-3, -7, -8 and -9 when compared with wild-type cells (Fig. 2a). Conversely, reintroduction of Rb into Saos-2 cells (an *Rb*-null, *p53*-null human osteosarcoma line) repressed caspase expression to almost undetectable levels (Fig. 2b). This repression correlates with the ability of Rb to bind to E2F, as a tumour-derived Rb mutant defective in this activity was unable to suppress caspase levels (RbΔ22; data not shown). Interestingly, the impact of Rb on

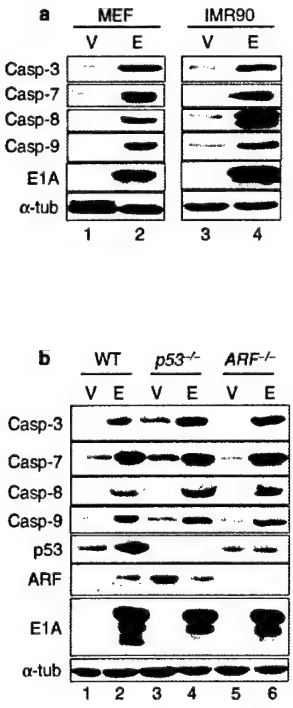

Figure 1 **E1A induces caspases independently of ARF and p53.** The levels of caspase proenzymes were assessed in cells transduced with a control retrovirus (V) or a retrovirus expressing *E1A* (E) by immunoblotting using antibodies directed against the indicated proteins. α-tubulin (α-tub) was used as a loading control. **a**, Casp-3, -7, -8 and -9 levels in MEFs and IMR90 cells. **b**, Casp-3, -7, -8 and -9 levels in early-passage wild-type, $p53^{-/-}$ and $ARF^{-/-}$ MEFs.

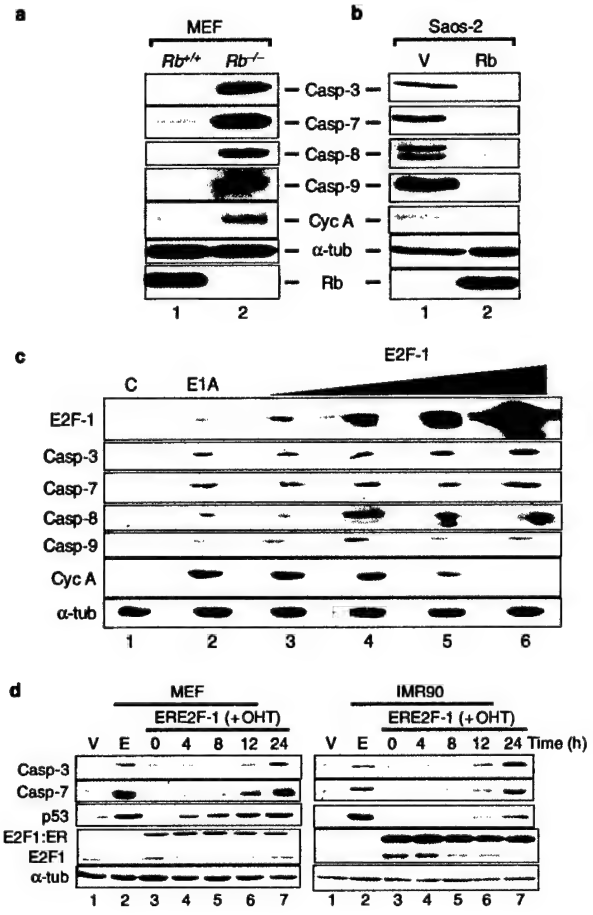

Figure 2 **Rb controls caspase expression.** Casp-3, -7, -8, -9 and cyclin A (Cyc A) expression levels were compared in wild-type ($Rb^{+/+}$) and $Rb^{-/-}$ MEFs (**a**) or in *Rb*-deficient Saos-2 cells after transfection with vector (V) or Rb constructs (**b**). *Rb* deficiency and equal loading were confirmed by examining expression of Rb and α-tubulin (α-tub), respectively. **c**, IMR90 fibroblasts were infected with adenoviruses expressing GFP, E1A and E2F-1 (triangle indicates increasing moi). Casp-3, -7, -8, -9, E2F-1, Cyc A and p53 levels were determined as above. The decline in *Cyc A* expression at high E2F levels presumably reflects the 'squelching' phenomenon often observed after using supra-physiological levels of transcription factors[36]. **d**, MEFs and IMR90 cells were infected with control (V), *E1A*-expressing (E) or *E2F-1*-expressing (ERE2F-1) retroviruses. At time-zero, medium containing 500 nM of 4-hydroxytamoxifen (OHT) was added and Casp-3, Casp-7 and p53 levels were determined at the indicated times. A mutant ERE2F-1 lacking the transactivation domain failed to induce caspases (data not shown).

expression of caspases paralleled the effects on expression of cyclin A (Fig. 2), a component of the cell-cycle machinery.

As loss of Rb can promote apoptosis through unrestrained E2F activity[2,7], we asked whether enforced expression of E2F could also induce caspase expression. E2F-1, the E2F family member most closely associated with apoptosis, was introduced into IMR90 cells using increasing amounts of a recombinant adenovirus. Caspase expression was determined 36 h later. As controls, parallel cultures were infected with adenoviruses expressing either green fluorescent protein (GFP; control) or E1A. Expression of E2F-1 was sufficient to induce caspase expression, even when produced at levels comparable to endogenous E2F in cells expressing E1A (Fig. 2c, compare lanes 2 and 3). With the exception of Casp-8, higher E2F-1 levels did not induce further increases in caspase expression (Fig. 2c, lanes 3–6). This effect was not specific to E2F-1 or to adenovirus-mediated gene delivery, as preliminary studies suggest that E2F-2 and -3 can also increase caspase expression (Z.N. & S.W.L., unpublished observations). Furthermore, both IMR90 cells and MEFs stably expressing a well-characterized E2F-1/oestrogen-receptor fusion construct[19] (ERE2F-1) accumulated caspase proteins in response to treatment with tamoxifen, an oestrogen-receptor ligand (Fig. 2d). Interestingly, the impact of E2F-1 on caspase expression correlates well with its ability to induce cyclin A, a *bona fide* E2F target-gene[7] (Fig. 2b, c).

Caspases can be direct transcriptional targets of E2F. E2F-1 directly controls the expression of genes involved in cell-cycle progression and S phase entry[7]. To determine if E2F regulates caspase transcription, we examined *caspase* mRNA expression in IMR90 cells expressing E1A or ERE2F-1 (in the presence and absence of tamoxifen) by northern blotting. Both E1A and E2F-1 increased expression of *caspase* mRNA 5–15-fold when compared with controls (Fig. 3a, compare lanes 1 and 2 for E1A and lanes 3 and 4 for E2F-1), which is consistent with cDNA microarray studies using cells expressing E1A (Z.N. & S.W.L., unpublished observations) or E2F-1 (ref. 20). The fact that E1A deregulates endogenous E2F activity and induces *caspase* mRNA expression implies that caspases are physiological targets of E2F-1. In further support of this view, the level of caspase expression was low in quiescent cells and higher as cells entered S phase, precisely paralleling the expression profile of cyclin A (Fig. 3b). However, the upregulation was less pronounced for Casp-8 and -9 when compared with Casp-3 and -7, suggesting that sig-

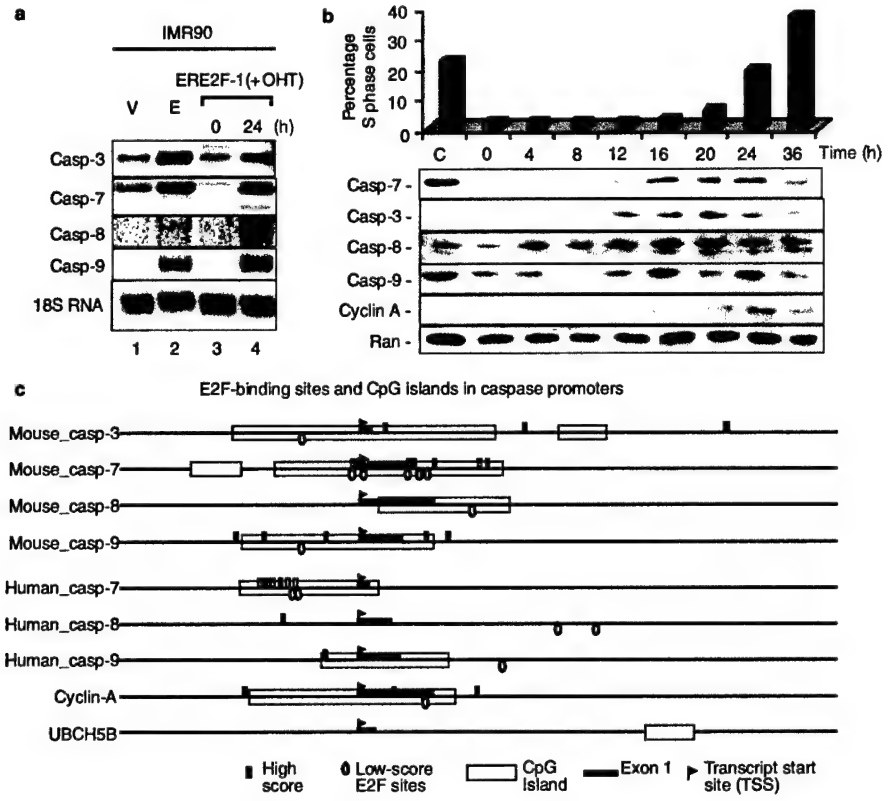

Figure 3 **Caspases display characteristics of E2F target-genes. a**, Northern blotting of total RNA isolated from IMR90 cells infected with the empty vector (V), *E1A* (E) or *ERE2F-1*-expression constructs using probes specific for the indicated caspase. 18S RNA was used as a loading control. E2F1 was induced by incubating cells with 500 nM OHT for 24 h. **b**, Expression of caspases was determined by immunoblotting in control (C) and serum-starved cells ($t = 0$), and at various times after serum addition. The percentage of cells in S phase at various times is shown. *Ran* expression is shown as a loading control. *Caspase 3* and *caspase 7* mRNAs were also elevated in growing cells when compared with arrested cells (data not shown). **c**, The genomic regions spanning 1000 bp upstream of the predicted caspase transcriptional start site and 500 bp into the first intron of mouse *casp-3, -7, -8, -9*, human *casp-7, -8, -9*, *Cyclin A* and *UBCH5B* were retrieved from genome databases and analysed for potential E2F-binding sites using SiteScan. Predicted E2F-binding sites for caspase genes are presented in this schematic diagram. Squares and dots represent high-score and low-score sites, respectively (see Supplementary Information Table).

nalling caspases require more sustained E2F activity to respond.

To further evaluate whether caspases are E2F target-genes, we investigated whether caspase promoters contain predicted E2F-binding sites using the SiteScan program, which identifies E2F-binding sites on the basis of consensus sequence and additional contextual motifs[21] (see Supplementary Information Table). Caspase genomic regions were assembled from sequence information in public and private databases before analysis using SiteScan. Each of the caspase promoters had features that have been observed in previously characterized E2F-responsive promoters, including at least one predicted E2F-1-binding site (Fig. 3b; Supplementary Information Table). As expected, SiteScan also identified the well-characterized E2F sites in the cyclin A promoter and did not identify sites in housekeeping genes, such as the ubiquitin-ligase enzyme, UBCH5B, which is not regulated by E2F.

If caspase genes are direct transcriptional targets of E2F, then E2F proteins should physically associate with these promoters when caspase expression is high and caspase promoter sequences should confer E2F responsiveness to heterologous genes. To verify binding of E2F-1 to caspase promoters *in vivo*, we used chromatin immunoprecipitation (ChIP)[22], which allows the detection of proteins bound to specific regions of DNA. We focused on the binding of E2F-1 to the *casp-7* promoter, which has an aggregate of putative E2F-1 sites (Fig. 3c). Vector- and E1A-expressing IMR90 cells were fixed in formaldehyde to crosslink proteins to the DNA. After sonication to generate ~500-base pair fragments of genomic DNA, E2F-1:DNA complexes were immunoprecipitated using an antibody against E2F-1. The DNA was released from immunoprecipitates and analysed by quantitative PCR using primers that flanked the putative E2F-binding elements.

casp-7 promoter sequences were readily detected in immunoprecipitates from *E1A*-expressing cells (Fig. 4b, top, lane 2). As controls, the same procedure amplified sequences corresponding to the *bona fide* E2F site in the cyclin A promoter, but not from the 3′ end of the *cyclin A* gene, which lacks E2F sites (Fig. 4a, and Fig. 4b, lanes 1 and 2). Although *casp-7* sequences were not amplified from normal cells (Fig. 4b, lane 1), it is possible that E2F-1 is bound to the promoter at low levels or in a manner that is not recognized by the antibody. Nevertheless, E2F-1 is bound to the *casp-7* promoter in cells prone to apoptosis.

To determine whether the *casp-7* promoter was responsive to E2F, a genomic fragment containing the predicted E2F sites (sequences −546 to +86 upstream of the transcriptional start site) was isolated and subcloned into a luciferase reporter plasmid (Casp-7-luc). The construct was then cotransfected into *p53*-deficient Saos-2 cells with either an empty vector (LPC), or increasing

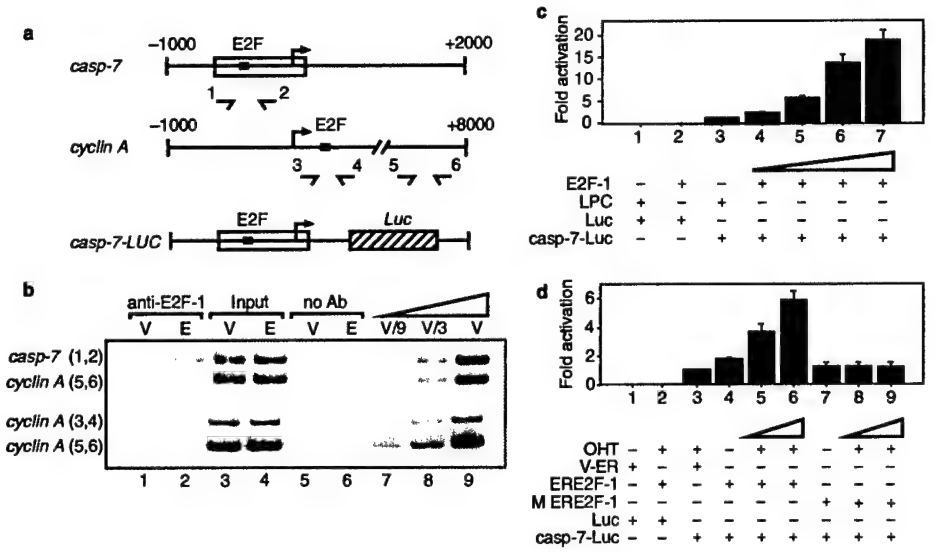

Figure 4 **Caspase 7 is a direct E2F-1 target. a**, Schematic diagrams depicting genomic regions of human *casp-7* and *cyclin A* genes, as well as the *casp-7*-luciferase construct. Open box represents the putative *casp-7* promoter region cloned into the luciferase reporter plasmid. Numbers correspond to primers with homology to the indicated sites (see Supplementary Information Table). **b**, *In vivo* detection of promoter occupancy by E2F using ChIP. Chromatin was prepared from IMR90 cells infected with control vector (V) or *E1A*-expressing (E) retroviruses and immunoprecipitated with antibodies specific to E2F-1. Duplex PCR amplification was performed on corresponding templates using the indicated primer sets. Input corresponds to PCR reactions containing 0.5% of total chromatin used in immunoprecipitation reactions. Parallel immunoprecipitation without antibody failed to yield detectable signals after equivalent autoradiographic exposure. Equal loading of templates in E1A and vector samples is shown in the input lanes, and a titration of DNA template was performed to demonstrate the linearity of PCR amplification. **c**, Luciferase reporter assays using Saos-2 cells cotransfected with empty vector (LPC) or E2F-1 plasmid in addition to a luciferase reporter plasmid containing the *casp-7* promoter (casp-7-Luc) or empty luciferase vector (Luc). The presence (+) or absence (–) of particular plasmids in the transfection is indicated and the triangle represents increasing amounts of *E2F-1* plasmid (20 ng, 100 ng, 500 ng and 1 μg, respectively). **d**, Luciferase experiments were performed exactly as in **c**, except that the reporter plasmids were cotransfected with empty vector (V-ER), ERE2F-1 or mutant ERE2F-1 (M ERE2F-1). Here, the triangles represent increasing concentrations of the ER fusion construct (500 ng and 1 μg, respectively). 500 nM OHT was added for 12 h to activate E2F-1.

concentrations of E2F-1 expression plasmid (Fig. 4c) and a normalization control. Luciferase activity was plotted as the fold induction relative to basal luciferase activity (that is, luciferase activity in cells receiving the reporter plasmid but no exogenous E2F-1). The results indicate that E2F-1 can induce an almost 18-fold increase in reporter activity over basal levels. We also cotransfected Saos-2 cells with the reporter construct and a plasmid encoding the aforementioned ERE2F-1 or the ERE2F-1 mutant. As expected, the *casp-7* promoter was responsive to E2F-1 after addition of tamoxifen, resulting in a sixfold increase in signal over basal levels (Fig. 4d). In contrast, the reporter was unresponsive to the ERE2F-1 mutant, even in the presence of tamoxifen (Fig. 4d, compare lane 7 with lanes 8 and 9). Taken together, our data demonstrate that E2F-1 transactivates the *casp-7* promoter. Given the coordinate regulation of Casp-2, -3, -7, -8 and -9 by E2F and the existence of E2F sites in the all caspase promoters examined, we suspect the other caspases are also direct targets of E2F.

Cells with high caspase levels are sensitized to apoptosis after release of cytochrome *c*. The fact that E1A utilizes the Rb–E2F pathway to induce apoptosis and caspase expression implies that high caspase levels facilitate oncogene-induced cell death. Accordingly, wild-type MEFs expressing *E1A* are sensitized to diverse apoptotic stimuli, including serum withdrawal and adriamycin treatment (activators of the Casp-9 pathway[23]) and the death ligand tumour necrosis factor α (TNF-α, an activator of the Casp-8 pathway[24]; Fig. 5a). However, accumulation of caspases is not sufficient to induce apoptosis, as *p53*-null cells expressing *E1A* are resistant to serum withdrawal and adriamycin treatment[25] but express elevated caspase levels, although they still respond to TNF-α. Therefore, caspase accumulation may potentiate cell death in the presence of an apoptotic stimulus.

Together with previous reports, our results indicate that E2F targets the apoptotic programme at multiple levels: for example, E2F can facilitate caspase activation through p53-dependent signals, resulting in mitochondrial release of cytochrome *c*, while simultaneously increasing caspase expression through a direct mechanism that is independent of p53. This model predicts that enforced cytoplasmic delivery of cytochrome *c* should bypass the requirement for p53 during oncogene-induced apoptosis, allowing the sensitization effect of elevated caspase expression to be uncoupled from the upstream processes required for caspase activation (see Fig. 5d). To test this hypothesis, we conducted two complementary experiments. First, we examined the ability of Bax — a pro-apoptotic member of the Bcl-2 family that functions downstream of p53 to facilitate cytochrome *c* release[25,26] — to potentiate apoptosis in $p53^{-/-}bax^{-/-}$ cells expressing *E2F*. Second, we microinjected cytochrome *c* directly into early passage $p53^{-/-}$ and $p53^{-/-}Rb^{-/-}$ MEFs. In both settings, the absence of p53 eliminated any p53-dependent component from our analysis, whereas enforced E2F expression or the absence of Rb provided a stimulus to increase caspase expression.

As predicted, cells that co-express E2F-1 and Bax underwent substantially more apoptosis when compared with cells expressing either E2F-1 or Bax alone (Fig. 5b). Moreover, at all cytochrome *c* concentrations tested, $p53^{-/-}Rb^{-/-}$ cells were significantly more sensitive to apoptosis than $p53^{-/-}$ cells (Fig. 5c). Of note, E2F-expressing cells were also more sensitive to cytochrome *c*-induced apoptosis when compared with controls (M.J. and D.B., unpublished observa-

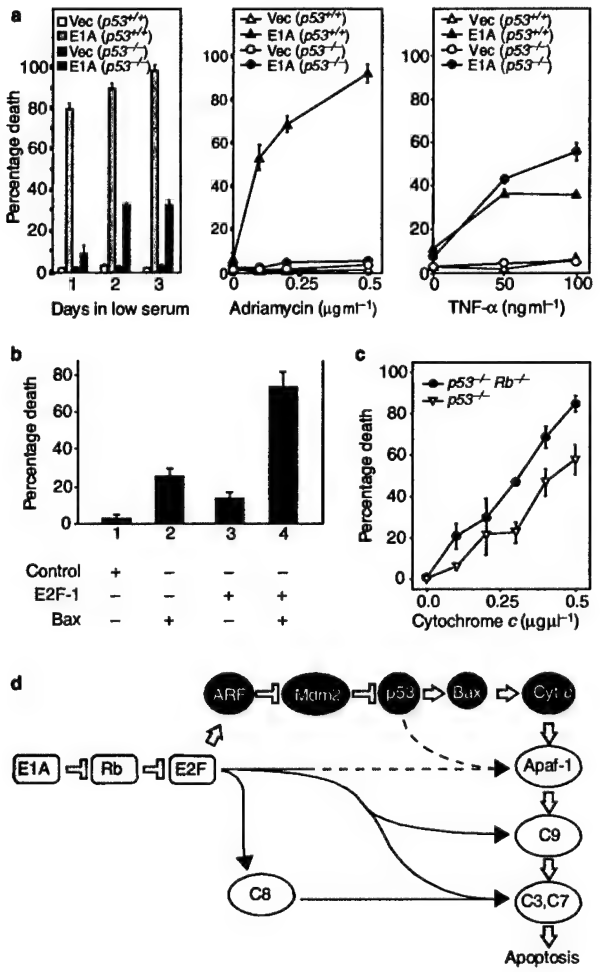

Figure 5 **Loss of Rb sensitizes cells to apoptosis after cytochrome *c* release. a**, $p53^{+/+}$ and $p53^{-/-}$ MEFs expressing a control vector (Vec) or E1A were incubated in the presence of 0.1% serum, adriamycin or TNF-α and the percentage of dead cells was determined at 24 h or at the indicated time. **b**, $p53^{-/-}bax^{-/-}$ MEFs expressing control vectors, Bax, E2F-1 or co-expressing Bax and E2F-1 were produced by gene transfer using a *bax*-expressing retrovirus and an *E2F-1*-expressing adenovirus. Cell death was assessed 36 h after adenovirus infection. The presence (+) or absence (–) of the particular genes is indicated. **c**, Cytochrome *c* was microinjected into $p53^{-/-}$ or $p53^{-/-}Rb^{-/-}$ MEFs at the indicated concentration. After 1 h, the injected cells were scored for apoptosis using morphological criteria. FITC–dextran was co-injected with cytochrome *c* to facilitate identification of injected cells. Each data point represents a total of 85–225 cells and the data represents the mean and standard deviation of three experiments. **d**, A model for oncogene-induced apoptosis involving both p53-dependent and p53-independent pathways. E2F signalling indirectly to p53 facilitates caspase activation and simultaneously produces increases in caspase expression. These two pathways function in concert to ensure efficient cell death. The dashed line is extrapolated from previous work suggesting that Apaf-1 is a transcriptional target of both E2F-1 and p53 (ref. 16). Both E2F-1 and p53 have additional targets not shown on this scheme, all of which may function in concert to produce efficient cell death.

tions). Given that cytochrome *c* functions late in the apoptotic programme, our results strongly suggest that E2F-mediated increases in caspase expression are responsible for this sensitization effect.

Discussion

In summary, we show that deregulation of the Rb–E2F pathway by E1A, loss of Rb or enforced E2F expression coordinately increases caspase expression, at least in part, through a direct transcriptional mechanism. Although not sufficient for apoptosis, high levels of signalling caspases may increase the probability that a death-inducing signal overcomes endogenous caspase inhibitors (for example, inhibitor of apoptosis (IAP) proteins) to trigger a caspase cascade, whereas high levels of effector caspases may ensure that apoptosis proceeds efficiently once initiated. Hence, p53-generated signals resulting in caspase activation (for example, cytochrome *c* release from mitochondria) cooperate with p53-independent increases in caspase expression to induce apoptosis (Fig. 5d). Alternatively, increases in caspase expression — particularly Casp-8 — may sensitize cells to death-inducing ligands[27,28]. Together, our results may help explain the ability of E2F-1 to cooperate with p53 in apoptosis[29] and the increased sensitivity of oncogene-expressing cells to pro-apoptotic cytokines, such as TNF-α[30]. More importantly, they imply that oncogene-induced apoptosis proceeds through a highly coordinated series of events, ensuring that cell death is efficiently executed.

Oncogene-induced apoptosis limits the transforming potential of excessive mitogenic signalling[1,31], but precisely how cells 'sense' aberrant proliferation has been unclear. Here, we have shown that this can be achieved, in part, by coupling cell-cycle progression to caspase expression through E2F. Hence, E2F transmits mitogenic signals to the cell-cycle machinery by directly regulating the transcription of genes required for S phase entry and simultaneously upregulates the cell death machinery through a similar mechanism. As a consequence, cycling cells are primed for apoptosis should proliferation be perceived as aberrant. Together, our results demonstrate that oncogenes can coordinately upregulate caspase expression through a direct mechanism, providing an example of how apoptosis can be 'hardwired' to cell-cycle progression. □

Methods

Cells and gene transfer

IMR90, U2OS and Saos-2 cells were obtained from the American Type Culture Collection (ATCC). Primary MEFs from wild type, $p53^{-/-}$, $ARF^{-/-}$, $Rb^{-/-}$, $p53^{-/-}$ $Rb^{-/-}$ and $p53^{-/-}$ $bax^{-/-}$ mice were isolated and cultured as described[32]. IMR90 cells (expressing the ectopic retrovirus receptor[33]) and MEFs were infected with high-titre recombinant retroviruses expressing *E1A* (LPC-12S), *ER^{E2F-1}* (pBabeHAERE2F-1 (ref. 19)) and a transactivation-defective mutant of E2F-1 (pBabeHAERE2F-1(1–374)[16]) as described[32]. For ER constructs, E2F-1 activity was induced by the addition of 500 nM 4-hydroxytamoxifen (OHT; Sigma, St Louis, MO). *E1A* and *E2F-1* were also introduced into IMR90 cells using recombinant adenoviruses[33] at a multiplicity of infection (moi) of 100 plaque-forming units (pfu) $cell^{-1}$ for Ad-$E1A_{12}$ and Ad-GFP, and 3, 12.5, 50, and 200 pfu $cell^{-1}$ for Ad-E2F1. For reintroduction of *Rb* into Saos-2 cells, calcium phosphate transfection was performed using Rb (pSVE) and GFP (pBABE) expression plasmids at a ratio of 4:1, respectively. Two days after transfection, cells were sorted by FACS (Elite ESP; Coulter, Miami, FL) and the GFP-positive cells collected and lysed for immunoblotting. For *Bax/E2F-1* co-infection, $p53^{-/-}$ $bax^{-/-}$ MEFs were infected with high-titre *Bax* (pLPC-HABax) retroviruses. After selection, cells received either Ad-E2F1 or Ad-GFP (moi of 250) and viability was determined 36 h later using a trypan blue exclusion assay.

Protein and gene expression

Protein expression was assessed by separating 30 μg of total cell lysate on 10% polyacrylamide gels before immunoblotting. Blots were probed with antibodies directed against Casp-2 (dilution 1:250; 13951A, PharMingen, San Diego, CA), Casp-3 (1:1000; C76920, Transduction Laboratories, Lexington, KY), Casp-7 (1:1000; clone 1-1-10), Casp-8 (1:500; 97-7-15), Casp-9 (1:500; AAP-119, Stressgen, Victoria, BC), E1A (1:50; M58 (ref. 34)), p53 (1:1000; CM5, CM1, Novocastra, Newcastle, UK), ARF (1:1000; NB 200-106, Novus Biologicals, Littleton, CO), Rb (1:1000; 14001A, PharMingen), E2F-1 (1:100; KH95, Santa Cruz Biotechnology, Santa Cruz, CA), Cyclin A (1:200; BF683, Santa Cruz) or α-tubulin (1:2000; B-5-1-2, Sigma). Anti-mouse or anti-rabbit horseradish peroxidase (1:5,000; Amersham, Piscataway, NJ) were used as secondary antibodies. Proteins were visualized using the ECL detection system (Amersham). mRNA levels were assessed by northern blotting using 10 μg of total RNA (Ultraspec RNA system, Biotecx, Houston, TX).

Caspase promoter analysis

mRNA sequences of human and mouse caspases (NM_001227, NM_001228, NM_001229, NM_009810, NM_007611, BC006737 and NM_015733) were retrieved from NCBI nucleotide database and aligned with genomic sequences by local alignment program BLAST. First exons and their flanking regions, 1000 bp upstream promoter region and 500 bp downstream intron region, were then retrieved from the GenBank and Celera databases and analysed for potential E2F-binding sites with the SiteScan program (http://compel.bionet.nsc.ru/FunSite/SiteScan.html). SiteScan generated

composite transcriptional modules consisting of a binding site for E2F transcription factors and additional contextual motifs[21] (see Supplementary Information Table).

Functional analysis of caspase promoters

ChIP was performed as previously described[22] using an E2F-1-specific antibody (E2F-1 sc-193, Santa Cruz). DNA released from precipitated complexes was amplified using sequence-specific primers to detect *casp-7* promoter elements (Fig. 4, oligonucleotides 1 and 2). As controls, oligonucleotides corresponding to the indicated positions (Fig. 4, oligonucleotides 3–6) were used to amplify sequences within the *cyclin A* gene. Genomic fragments corresponding to the *casp-7* promoter were amplified by PCR from HeLa cells and cloned into the pGL3-Basic luciferase reporter vector (Promega, Madison, WI). U2OS or Saos-2 cells were transfected using FuGene 6 (Roche, Basel, Switzerland) with 2 μg of the reporter construct, 20–1000 ng of the E2F1 expression plasmid and 1.2 μg of the pRL-β-globin control plasmid (Promega). Cells were harvested 36 h after transfection. Luciferase activity was assayed using a luminometer and normalized to the transfection control. The sequences of oligonucleotides used in the ChIP assay and for amplifying the *casp-7* promoter are available from the authors on request.

Cell-cycle analysis

IMR90 fibroblasts were incubated in DMEM (Invitrogen, Carlsbad, CA) medium supplemented with a suboptimal concentration of foetal bovine serum (0.1%) for 4 days. Normal serum level (10%) was restored and cells harvested at 0, 4, 8, 12, 16, 20, 24 and 36 h after release from low serum. Nuclei from each time-point were stained with DAPI and analysed for DNA content by flow cytometry.

Microinjection

Cytochrome *c* (C-3131; Sigma), diluted in 0.5× PBS containing 2 mg ml^{-1} fluorescein isothiocyanate (FITC)–dextran (FD-20; Sigma) was injected into MEFs using an Eppendorf microinjector (5242) at the indicated concentrations, as described previously[15]. The cells were incubated for 1 h at 37 °C before scoring. Apoptosis was determined using blebbing and cell rounding as morphological markers, whereas FITC–dextran staining permitted identification of successfully injected cells.

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We thank I. Nevins and K. Helin for E2F-1 expression vectors and L. Faleiro for contributions to the early stages of this work. We also thank J. Pelletier for helpful discussions, and E. de Stanchina and other members of the Lowe laboratory for encouragement and support. We thank J. Duffy for help in preparing the figures. This work was supported by Department of Defence Breast Cancer Research Program predoctoral fellowships (Z.N. and I.P.) and also by a programme project grant CA13106 from the National Cancer Institute (Y.L. and S.W.L.), and NIH-HG01696 from the National Institutes of Health (R.V.D. and M.Q.Z.). Z.N. is a DOD-BCRP fellow. S.W.L. is a Rita Allen Scholar.

Correspondence and requests for material should be addressed to S.W.L.

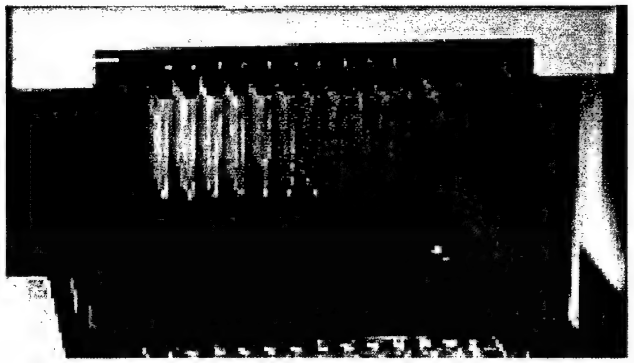
Supplementary Information accompanies the paper on www.nature.com/naturecellbiology.

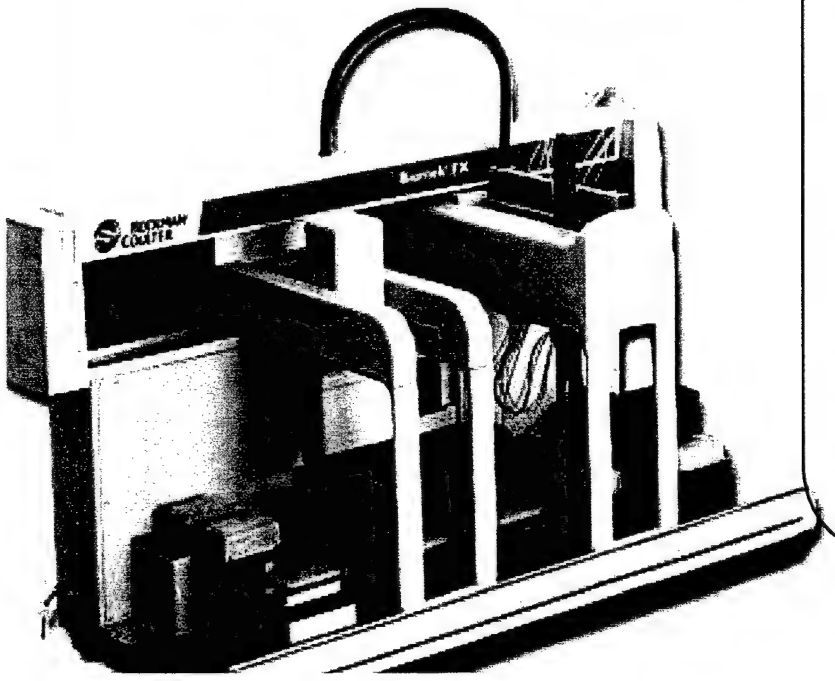
COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

Gene	Sequence of potential site	Positionrel. TSS	Score of sequence, q	Score of context	Value
m casp 3	aACCCGCGCGAGGg	-247 to -236	(+) 0.72	0.44	low
	aGCTGGCGCTCTTt	102 to 113	(+) 0.81	2.45	high
	tTCTTGCGGAGAGc	689 to 700	(+) 0.76	8.31	high
	gCTTGGCTCCCTTc	1533 to 1544	(+) 0.71	1.83	high
m casp 7	gCTTCCCGCGCGAg	-35 to -24	(+) 0.81	0.76	low
	tTCCCGCGCGAGCt	-33 to -22	(+) 0.78	7.53	high
	cTCGCGCGGGAAGc	-23 to -34	(-) 0.72	6.12	high
	gCTTCCCGCGCGAg	-22 to -11	(+) 0.81	4.98	high
	aGCTCGCGCGGGAa	-21 to -32	(-) 0.82	5.67	high
	tTCCCGCGCGAGCt	-20 to -9	(+) 0.78	7.19	high
	cTCGCGCGGGAAGc	-10 to -21	(-) 0.72	12.6	high
	aGCTCGCGCGGGAa	-8 to -19	(-) 0.82	12.67	high
	tTCTCCCGCGGCGg	-7 to 4	(+) 0.76	4.37	high
	cCGTAGCGCGCGTt	10 to 21	(+) 0.79	4.26	high
	gTAGCGCGCGTTCc	12 to 23	(+) 0.77	8.66	high
	cGTTCCCGCTCGCc	20 to 31	(+) 0.78	1.46	high
	gGAACGCGCGCTAc	24 to 13	(-) 0.71	6.4	high
	cCATCGCGGCTTTc	202 to 213	(+) 0.73	3.17	high
	cGATGGCGGCCCAa	206 to 195	(-) 0.74	0.58	low
	aGCTCGCGGAAGAg	234 to 223	(-) 0.74	7.43	high
	gGGACGCGCGGGAg	253 to 242	(-) 0.70	4.39	high
	tCTGGGCGGGGACg	261 to 250	(-) 0.74	0.45	low
	gTTCTGCGAGCCTg	291 to 280	(-) 0.73	0.43	low
	cTTCCGCGCCACAg	319 to 308	(-) 0.86	1.26	high
	cTTAGGCGCTGCCg	498 to 509	(+) 0.83	3.83	high
m casp 8	gTTTGGCTCAGGAc	468 to 479	(+) 0.76	0.92	low
m casp 9	gCTTAGCGGTAGTt	-512 to -523	(-) 0.80	2.82	high
	gTAGGGCGGGATTg	-404 to -393	(+) 0.71	3	high
	gCGTGGCGAAGGGg	-251 to -240	(+) 0.71	0.71	low
	aGTGAGCGCGAGTc	-144 to -133	(+) 0.78	1.41	high
	tTTTTCCGCTTTGc	276 to 287	(+) 0.80	4.33	high
	tTGTGGCGAACTAa	377 to 366	(-) 0.78	5.38	high

Gene	Sequence of potential site	Positionrel. TSS	Score of sequence, q	Score of context	Value
h casp 7	aTGGGGCGCTCGTg	-406 to -417	(-) 0.76	3.76	high
	aATCTGCGCTCTTt	-386 to -375	(+) 0.74	10.6	high
	aTTTCGCGGCGGCg	-385 to -396	(-) 0.89	5.29	high
	aCTTGGAGCGCGGc	-367 to -356	(+) 0.73	7.11	high
	tTGGAGCGCGGCCa	-365 to -354	(+) 0.73	8.88	high
	tTTTCCCGGAACAa	-334 to -323	(+) 0.78	11.65	high
	gTTTCCCGGGACAg	-293 to -304	(-) 0.80	10.24	high
	gCTGTGCGCGTTCt	-281 to -270	(+) 0.76	0.21	low
	tTCTCCCGCGCGCg	-271 to -260	(+) 0.77	6.48	high
	cTCCCGCGCGCGGg	-269 to -258	(+) 0.77	2.97	high
	cCCGCGCGCGGGCt	-267 to -256	(+) 0.70	2.49	high
	cCCGCGCGCGGGAg	-257 to -268	(-) 0.71	2.55	high
	aGCCCGCGCGCGGg	-255 to -266	(-) 0.71	5.51	high
h casp 8	tATTTGCGTTTTAa	-310 to -321	(-) 0.76	6.63	high
	tTTTAGCTCCGAGt	841 to 830	(-) 0.74	0.09	low
	cTCTGTCGCCTAGc	992 to 1003	(+) 0.75	0.83	low
h casp 9	gTGTTCCGCGGCTg	-145 to -134	(+) 0.73	6.61	high
	gTTCCGCGGCTGGt	-143 to -132	(+) 0.78	1.06	high
	tTTTGGCTGGTCTt	597 to 608	(+) 0.71	3.33	high
h Cyc A	tTTTTCCGCAGCCg	-175 to -186	(-) 0.79	0.73	low
	gCCTTGCGGGCTCc	404 to 415	(+) 0.72	1.05	high
	cTTTCGCGCTCTGc	646 to 657	(+) 0.97	1.9	high





Clone Collections

Replication

Primary PCR

Secondary PCR

Purification

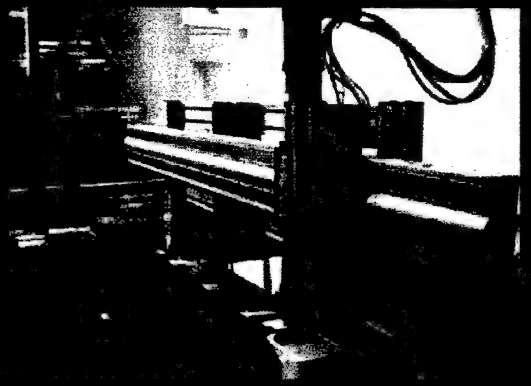
Reformatting

Printing

Gel Confirmation

APPENDIX B

Printing Protocols



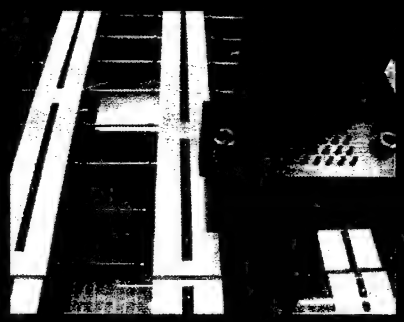
Arrayer Built in House- magnetic motors

Pins- Majer Precision stainless steel split pin

Spotting Solution 3XSSC

Printing Protocol*

- Environmentally Controlled Room
- Sonicate Pins
- Test with Salmon Sperm DNA in 3XSSC



COLD SPRING HARBOR LABORATORY

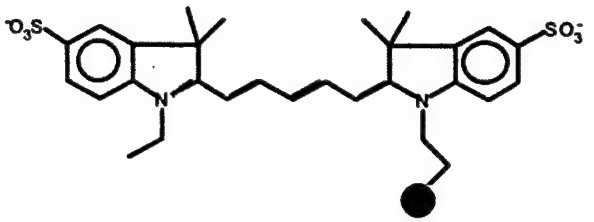
SYBR Green II Stain for Processing and Printing Quality Check

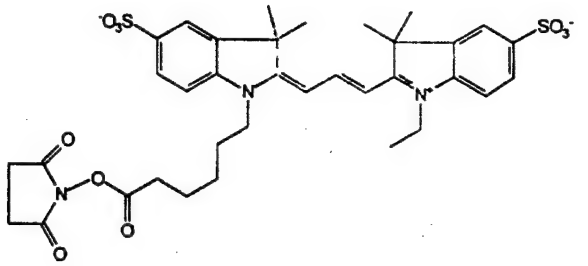
DNA less than 80ng/μl

DNA at or above 100ng/μl

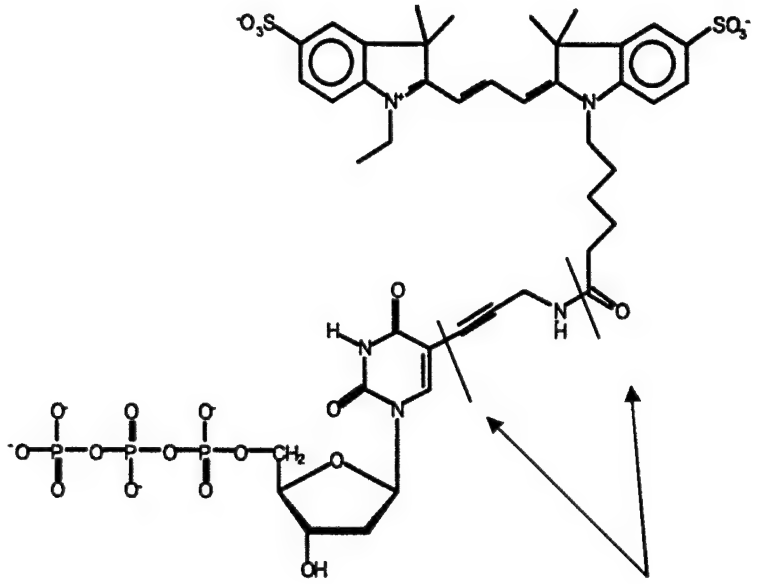
Labeling Dyes (fluorescing Cyanines)

Cy5-AP3-dUTP

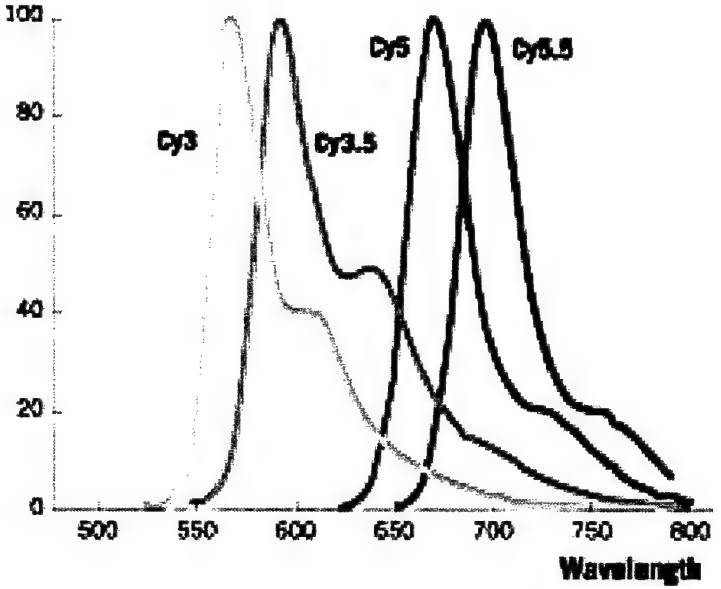




Cy3 monofunctional dye

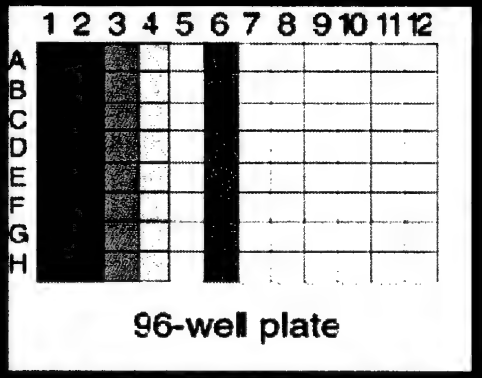


Cy3-AP3-dUTP



Potential Problems in Processing

Plate Rotation During Processing



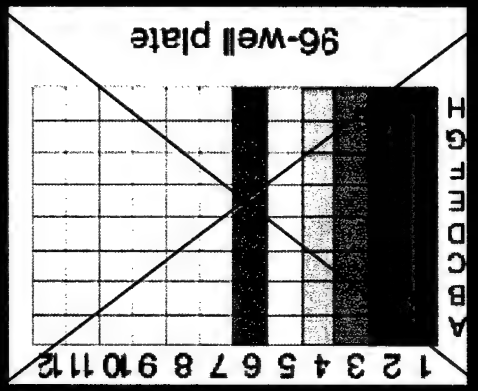
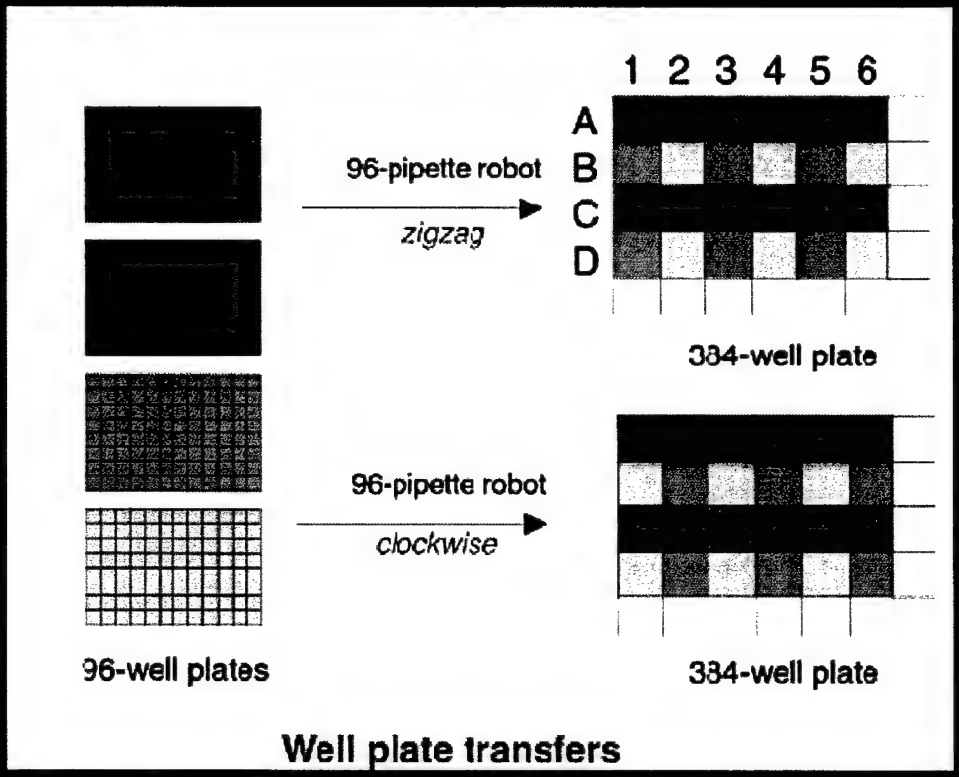


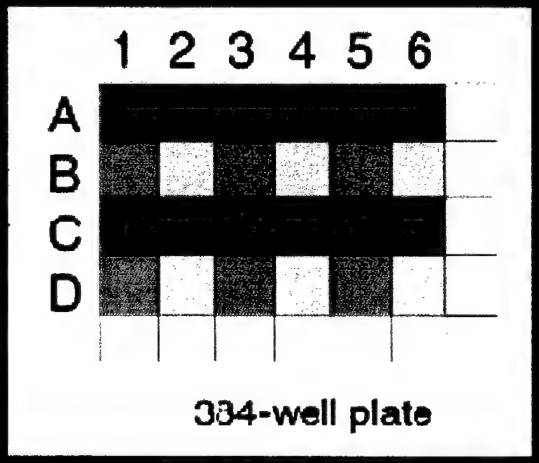
Plate Rotation Rotation During Reformatting

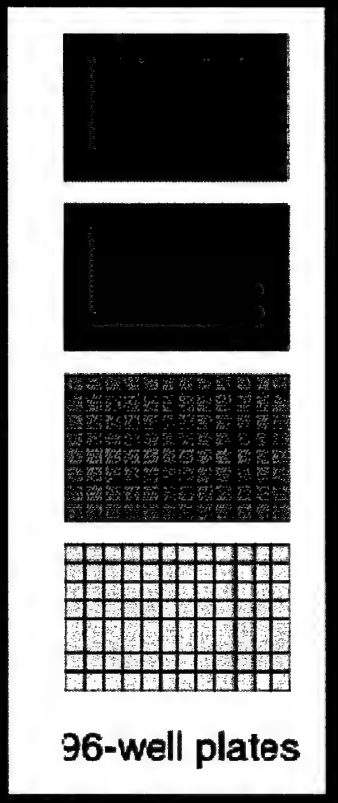


What Quality Controls Do We Have For Processing and Printing?

Quality Control Checks for Plate Rotations

- Sequencing A1, A2, B1, B2





- *Lambda* and *Adenovirus DNA*

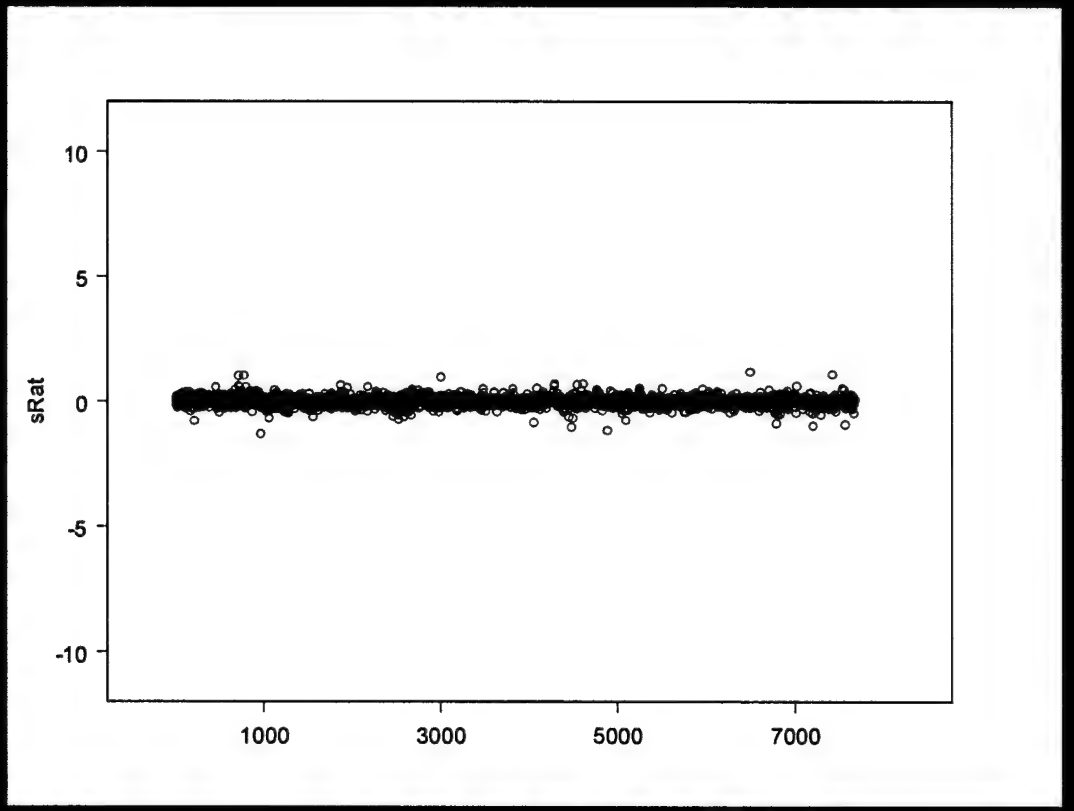
These two types of DNA are placed into locations of 384 well plates which create a pattern when printing.

A hybridization with *Lambda* and *Adenovirus* DNA spiked into the labeling reaction will confirm that all plates were loaded in the correct order.

Serves as a control for DNA carryover.

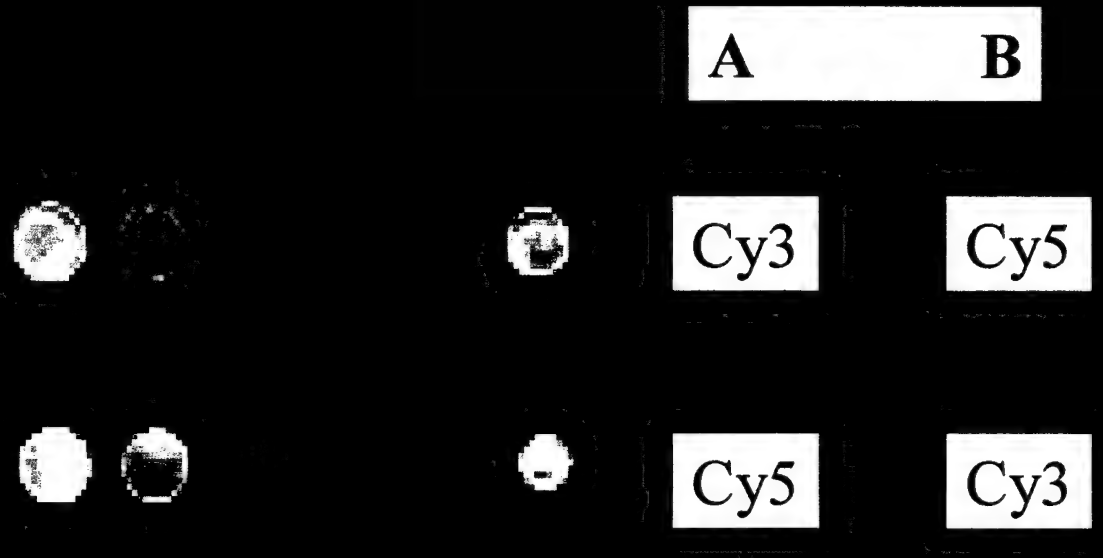
β actin
Poly A
Cot 1
Plant 3
Plant 2
Plant 1
SSC
1:2
2:1
1:1
3 cm
1.5 cm

Self to self zn43 m2n62

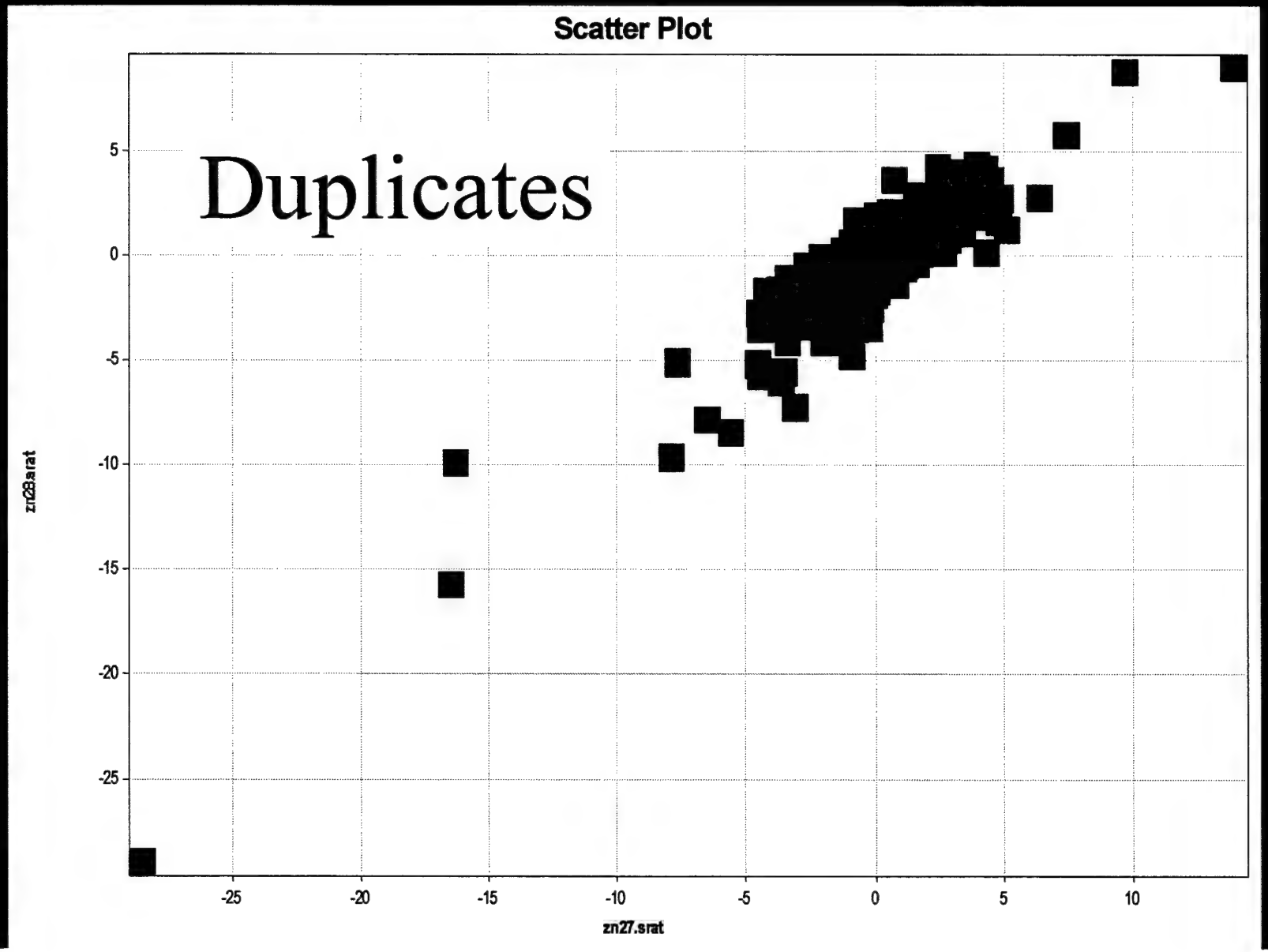


Control 2

Color reversal



Control 3


Scatter Plot
Duplicates
5
0
-5
-10
-15
-20
-25
zn28.srat
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-20
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-10
-5
0
5
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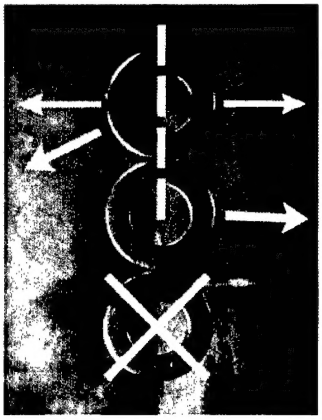
zn27.srat



APOPTOSIS

Divide or die

Cell proliferation and cell death might seem like opposing functions, but oncogenes such as *E1A* and *MYC* are able to initiate both. Apoptosis is thought to be a safety mechanism, and is induced when the oncogenic signal to proliferate is recognized as excessive, and hence likely to cause tumorigenesis. The mechanisms by which oncogenes induce cell death have not been clearly established. Zaher Nahle, Scott Lowe and colleagues have investigated this phenomenon using E1A, and have found that the E2F transcription factor, which promotes replication and hence proliferation, has a key role in coordinating these processes.

The authors first investigated whether overexpression of *E1A* in mouse embryo fibroblasts (MEFs) and normal diploid human fibroblasts (IMR90 cells) affected the protein levels of caspases — the effectors of cell death — and found that both initiator and effector caspases were upregulated by 5–15-fold. A similar increase was observed in cells deficient for either ARF or p53, so E1A must upregulate caspases through a p53-independent pathway.

One of the key targets of E1A is retinoblastoma (RB), and E1A mutants that are unable to inactivate RB do not upregulate caspases. Similarly, $RB^{-/-}$ MEFs expressed higher levels of caspases than wild-type cells. Introduction of wild-type *RB*, but not a tumour-derived mutant that can not bind E2F, into RB-deficient cells represses this caspase expression, and implicates the E2F family of proteins in this apoptotic pathway. In fact, expression of E2F1 is sufficient to induce this caspase induction.

So are caspases transcriptional targets of E2F1, or is the induction indirect? Northern blots showed that caspase mRNA was increased by 5–15-fold (similar to the protein levels) when either E1A or E2F1 was expressed in IMR90 cells. Caspase mRNA levels also increase as cells enter S phase, which coincides with the activity of E2F1 and the levels of cyclin A mRNA — a known E2F1 target. Analysis of caspase promoters provided further support that E2F could transcriptionally activate caspases, as several contain E2F1-binding sites, and chromatin immunoprecipitation experiments confirmed this — E2F1 precipitates from E1A-expressing cells that contained sequences from the caspase-7 promoter. The caspase-7 promoter was also able to drive transcription of the luciferase reporter gene — expression increased by almost 18-fold — when E2F1 was expressed.

But what is the physiological role of this caspase induction? It is not sufficient to induce apoptosis and, instead, seems to sensitize cells to apoptotic stimuli, such as serum withdrawal and adriamycin treatment. E2F1 is also known to activate cytochrome *c* release — a downstream event in the apoptotic pathway — by a p53-dependent pathway. The requirement for p53 in inducing apoptosis could be recapitulated, at least in part, by either introducing BAX — a proapoptotic protein that facilitates cytochrome *c* release — to $TP53^{-/-}$ $BAX^{-/-}$ cells expressing *E2F*, or by directly microinjecting cytochrome *c* into $TP53^{-/-}$ $RB^{-/-}$ cells. Under these conditions, caspase induction by E2F1 is able to enhance apoptosis, underscoring the cooperation of the p53-dependent and -independent pathways in inducing apoptosis.

So, the *E1A* oncogene coordinates division and death by using the same machinery — E2F — to initiate both processes. Whether other oncogenes operate in the same way remains to be determined.

Emma Greenwood, Senior Editor, Nature Reviews Cancer

References and links

ORIGINAL RESEARCH PAPER Nahle, Z. *et al.* Direct coupling of the cell cycle and cell death machinery by E2F. *Nature Cell Biol.* **4**, 859–864 (2002)

IN BRIEF

CELL SIGNALLING

A novel Epac-specific cAMP analogue demonstrates independent regulation of Rap1 and ERK.

Enserink, J. M. *et al. Nature Cell Biol.* **4**, 901–906 (2002)

Protein kinase A (PKA) is required for the cyclic AMP-induced activation of the extracellular signal-regulated kinase (ERK), but not for the cAMP-induced activation of the small GTPase Rap1 through the guanine nucleotide-exchange factors Epac1 and Epac2. Using a cAMP analogue as a tool to distinguish between PKA- and Epac-mediated effects, the authors found that the cAMP-induced regulation of ERK and the activation of Rap1 are independent processes.

NUCLEAR TRANSPORT

Karyopherins in nuclear pore biogenesis: a role for Kap121p in the assembly of Nup53p into nuclear pore complexes.

Lusk, C. P. *et al. J. Cell Biol.* **159**, 267–278 (2002)

Karyopherins — also known as importins or exportins, depending on the direction of transport — mediate nuclear transport through the nuclear pore complex (NPC). Karyopherin Kap121 interacts specifically with nucleoporin Nup53, and Lusk *et al.* now show that Kap121 targets Nup53 to the NPC. Nup53 is subsequently released from Kap121 by Nup170, which allows Kap121 to continue its movement through the NPC.

TELOMERES

A bulged stem tethers Est1p to telomerase RNA in budding yeast.

Seto, A. G. *et al. Genes Dev.* **16**, 2800–2812 (2002)

The RNA subunit of telomerase functions as the template for telomeric DNA synthesis, but it is not clear whether the RNA subunit has other functions. Here, Seto *et al.* report the identification of a conserved RNA bulged stem that is essential for telomerase function *in vivo* and that interacts with the telomerase regulatory protein Est1, which recruits or activates telomerase at the telomere.

GENE EXPRESSION

The SR protein SRp38 represses splicing in M phase cells.

Shin, C. & Manley, J. L. *Cell* **111**, 407–417 (2002)

SR proteins function in both constitutive and alternative pre-mRNA splicing. Shin and Manley now report the identification of an unusual member of the family — SRp38. Unlike other SR proteins that induce splicing, SRp38 acts as a splicing repressor that is activated by dephosphorylation during M phase. In mitotic cells splicing is inhibited by SRp38, which implies that cell-cycle-specific dephosphorylation of SRp38 is involved in gene silencing during mitosis.

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Emma Greenwood

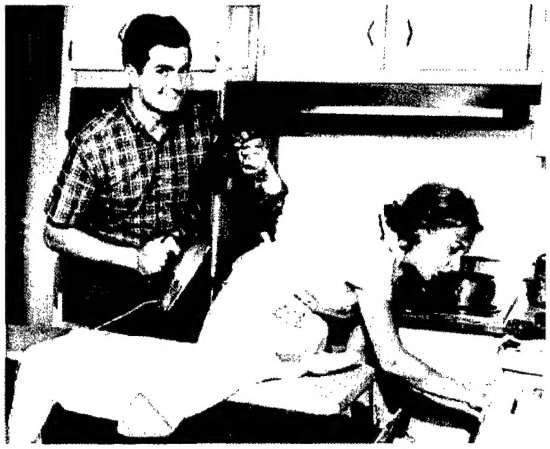
References and links

ORIGINAL RESEARCH PAPER Nahle, Z. *et al.* Direct coupling of the cell cycle and cell death machinery by E2F. *Nature Cell Biol.* **4**, 859–864 (2002)

FURTHER READING Trimarchi, J. M. & Lees, J. A. Sibling rivalry in the E2F family. *Nature Rev. Mol. Cell Biol.* **3**, 11–20 (2002)

WEB SITE

Scott Lowe's lab: http://www.cshl.org/public/SCIENCE/lowe.html



CHEMOTHERAPEUTICS

Opposites don't always attract

PC-SPES — a herbal preparation that is commonly taken by prostate cancer patients — interferes with cell-cycle arrest and apoptosis, but the precise mechanisms involved have been unclear. But now, Bonham and colleagues, in 6 November issue of *Journal of the National Cancer Institute*, show that PC-SPES might interfere with microtubule polymerization. The authors show how PC-SPES interacts with microtubule-modulating chemotherapeutic agents, such as paclitaxel, and discuss the implications that these findings have for patients.

To assess the mode of action of PC-SPES, the authors compared the gene expression profile of LNCaP prostate adenocarcinoma and six other cell lines before and after treatment with PC-SPES. After 48 hours of treatment, 319 transcripts were altered, many of which were involved in either cell cycling, immunity, cell stress or androgen regulation. However, the expression of one gene — α-tubulin, which has a crucial role in cell division — was shown to decrease in all seven cell lines tested. Further analysis by northern blotting showed that α-tubulin levels in LNCaP cells decreased by sevenfold after 48 hours, and immunofluorescence showed that the microtubules began to exhibit signs of disruption after just 8 hours of treatment with PC-SPES.

Further investigations revealed that this disruption was directly caused by a reduction in the rate and overall amount of tubulin polymerization. By contrast, α-tubulin levels were only marginally reduced in paclitaxel-treated LNCaP cells, microtubules were stabilized, and the rate and overall amount of tubulin polymerization was increased with this treatment. In addition, tumour growth was inhibited to a lesser extent when mice were treated with both PC-SPES and paclitaxel, compared with paclitaxel alone. This indicates that the tubulin dynamics induced by paclitaxel might be antagonistic to those caused by PC-SPES, and that PC-SPES attenuates the effects of paclitaxel.

So, although PC-SPES seems to be a beneficial 'complementary' therapy for prostate cancer patients, when taken in conjunction with conventional chemotherapeutics such as paclitaxel, the efficacy of treatment is reduced. Care should therefore be taken when using complex herbal preparations, about which little is known, in combination with more conventional therapies.

Sandra Clark

References and links

ORIGINAL RESEARCH PAPER Bonham, M. J. *et al.* Effects of the herbal extract PC-SPES on microtubule dynamics and paclitaxel-mediated prostate tumor growth inhibition. *J. Natl Cancer Instit.* **94**, 1641–1647 (2002)

Fall Festival Event Guide
Inside

Cops still looking for gunmen in drive-by
Page 3

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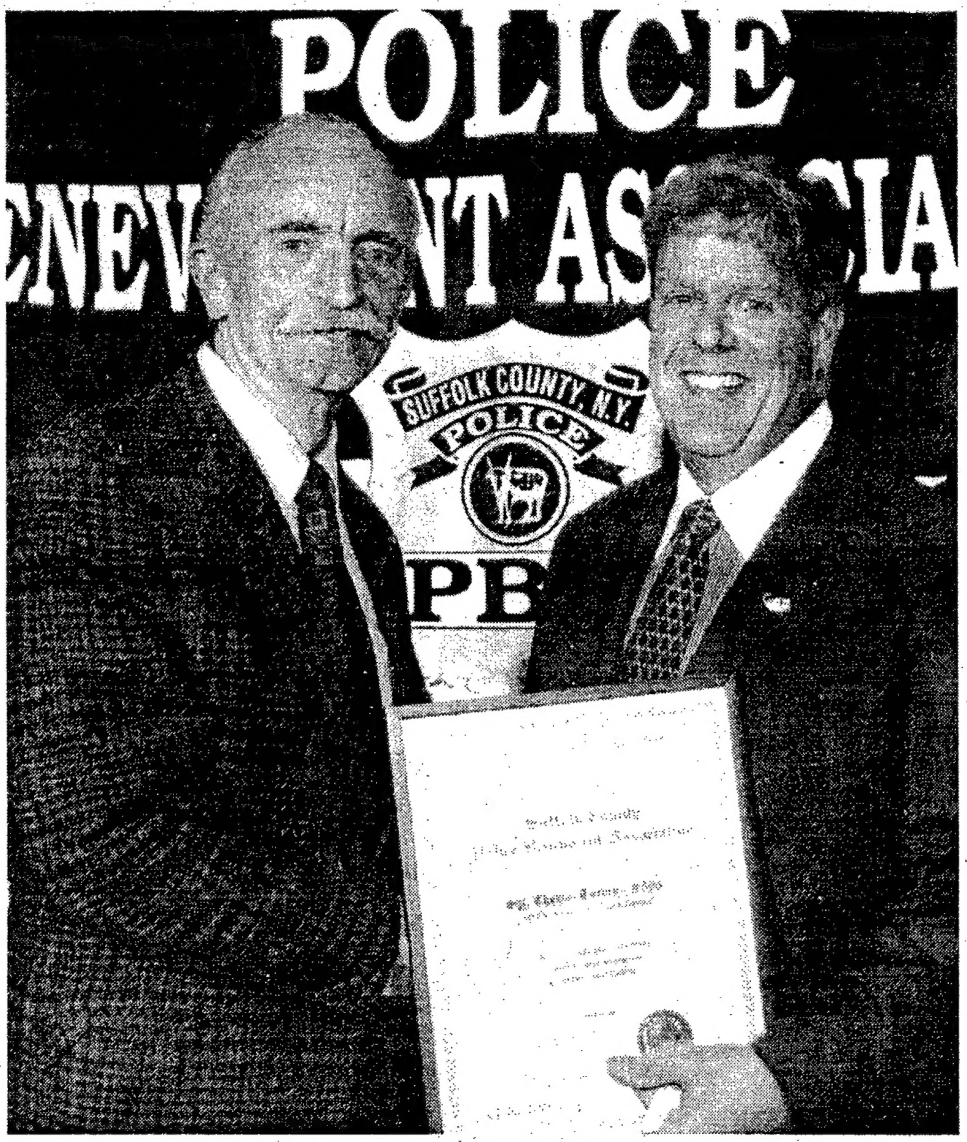
COLD SPRING HARBOR

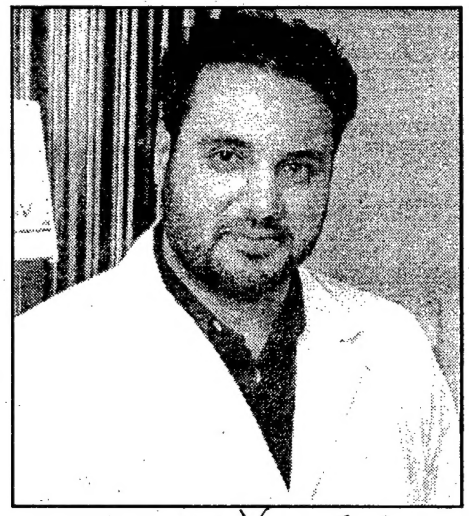
MISSION: POSSIBLE

CSH Labs top cancer researchers targeting means for cure

HAVE YOU HEARD ...



Suffolk County Police Benevolent Association President Jeff Frayler is pictured with Sergeant Tom Tierney of Greenlawn, at a recent retirement dinner hosted by the P.B.A. Sergeant Tierney was honored for 33 years of dedicated service.



Edwin Perez

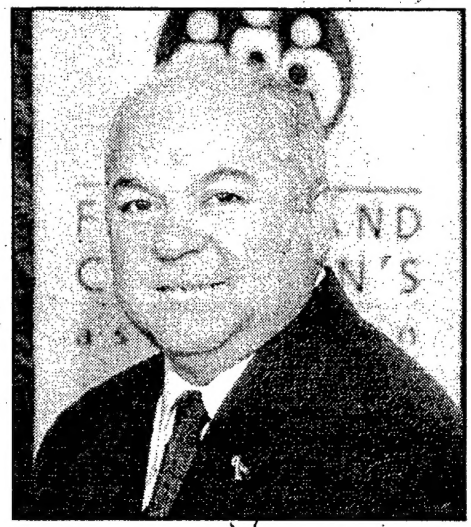
Edwin Perez of Huntington has joined the board of trustees of the nonprofit, nonsectarian human service agency, Family and Children's Association. He is a senior account executive with KeySpan's gas marketing and sales division.

A graduate of Bernard M. Baruch College with a BBA in marketing management, he is responsible for building alliances and managing relationships between the minority and women-owned business market segment.

"It is a pleasure to welcome Edwin Perez as a Family and Children's board trustee," said Dr. Richard P. Dina, agency president and CEO. "He brings much to the Family and Children's table, blending a strong business background with a deep knowledge of Long Island's problems and accomplishments, as well as a long-term relationship with minority communities that will enrich the work of this organization as we go forward together."

Huntington Station resident Zaher Nahle has received a three-year Department of Defense Breast Cancer Predoctoral Fellowship Award from the Congressionally Directed Medical Research Programs of the United States Army. Zaher's grant proposal for this highly competitive award ranked in the 96th percentile of all submissions and was rated "outstanding" by the scientific reviewing committee. The fellowship will support Zaher's cancer research at Cold Spring Harbor Laboratory, which aims at improving the understanding of the role and function of a major tumor suppressor pathway that is highly mutated in breast cancers.

Zaher is currently working on his Ph.D. at SUNY Stony Brook and Cold Spring Harbor Laboratory.



Zaher Nahle